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ORIGINAL ARTICLE

Phylogenetic, Demographic and Dating Analyses of *Bufo gargarizans* Populations from the Zhoushan Archipelago and Mainland China

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Abstract Relatively little is known about the relationship between *Bufo gargarizans* populations from Zhoushan Archipelago and nearby continental regions on the Pacific coast of eastern China. In this paper, 155 new specimens of *B. gargarizans* from Zhoushan Archipelago and adjacent continents and 71 published specimens of *B. gargarizans* from mainland China were studied. Phylogeographical and dating analyses of *B. gargarizans* were performed using mitochondrial DNA sequencing with a length of 1436 bp. A mtDNA tree that indicated seven major clades was obtained. The earliest split in the mtDNA tree corresponding to the divergence of populations from the western highland region occurred approximately 4.0 million years ago (mya). A subsequent clade occurred about 3.4 mya, with cladogenesis continuing toward the end of the Pleistocene. The continental clades were distributed in the western, central and northeastern regions of China. Zhoushan Archipelago clades consisted of two largely geographically overlapping subclades with the mtDNA divergence time of 0.73 mya. These results indicated there was extensive dispersal after vicariance. The *B. gargarizans* populations on Zhoushan Archipelago most probably originated from populations in nearby eastern continental regions of China. It was concluded that geological uplifting during the Pliocene and several sea-level changes in Pleistocene might have influenced the divergence and population demographical history of this species.

Keywords genetic structure, island phylogeography, mitochondrial DNA, *Bufo gargarizans*

1. Introduction

Islands are key regions for biogeographical studies. To date, researches in Europe, the Mediterranean and the Atlantic Ocean (Rijsdijk *et al*., 2014; Rodríguez *et al*., 2013; Szarowska *et al*., 2016), North America (Schoville and Roderick, 2009), Africa (Everson *et al*., 2016), South America (Gama *et al*., 2016) and Oceania (Andersen *et al*., 2015; Lohman *et al*., 2011) have indicated that the sea-level change due to climate change in the Pleistocene causing the formation and disappearance of refugia. It is one major factor influencing the evolution of animals on islands.

Zhoushan Archipelago, in the East China Sea at the entrance to Hangzhou Bay (Figure 1), is the largest archipelago of China. It has been subject to several sea-level fluctuations due to tectonic movements during the Zhe-Min Uplift and the glacial-interglacial cycles in the late Pleistocene. Therefore, it is a potentially interesting region for testing phylogeographic hypotheses. In addition, the East China Sea shelf basin has experienced at least three large sea-level fluctuations since the late Pleistocene (Li, 2008). During glacial periods, Zhoushan Archipelago was connected to the mainland by a land bridge. However, during interglacial periods, the land bridge was flooded. During the Holocene, sea levels rose dramatically, and Zhoushan Archipelago were formed about 7000–9000 years ago. These changes influenced the history of population segregation and exchange, and led to present-day distribution patterns of animal populations.

Recently, a few studies of biodiversity on China's islands (*i.e.*, including species surveys, taxonomic studies

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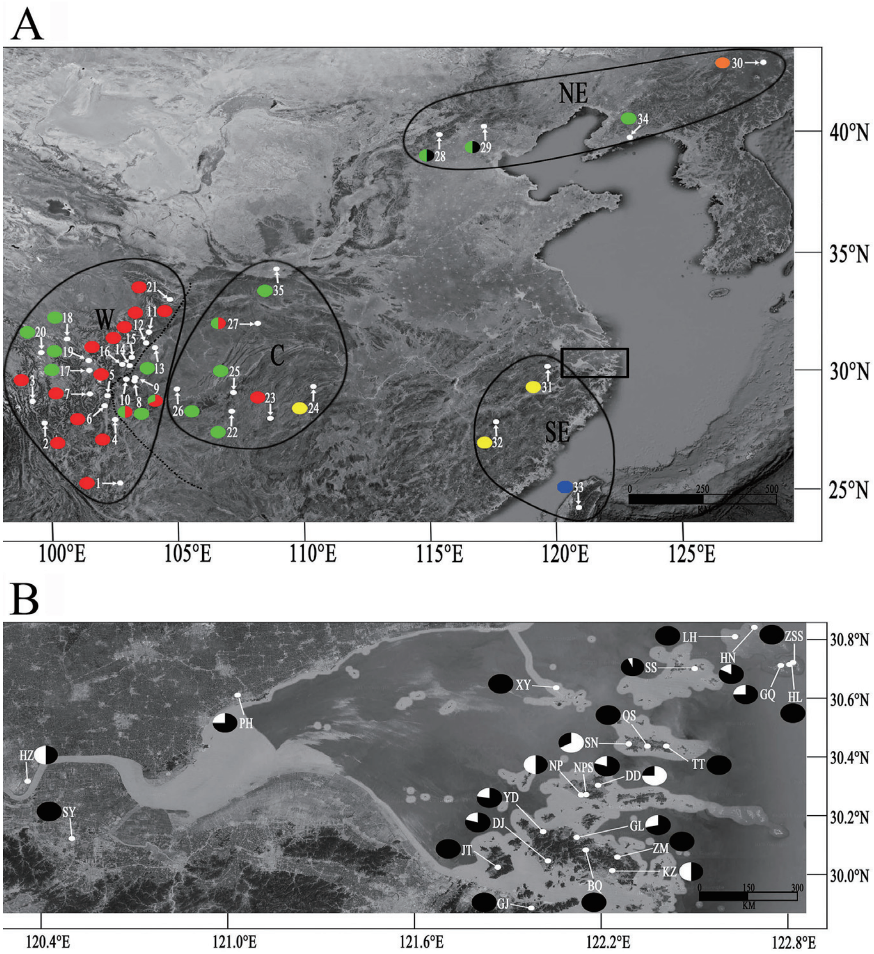


Figure 1 Map showing sampled localities of *Bufo gargarizans* (numbered white dots). Site names were in the Appendix I. Solid colored circles represented the *B. gargarizans* clades identified by phylogeographic analyses (each color represented one clade). A: A total of 34 sites from which 71 individuals were sampled. This figure was from Fu *et al.* (2005) with modification. All sequences including ND1–ND2 and Cytb–CR fragments of the 71 individuals published in their work were included here. B: A total of 24 newly added sites mainly in Zhoushan Archipelago and nearby continent (corresponding to the rectangular area in A) where 155 individuals were sampled. Populations at the left side of the dashed line in A were from highlands and others were from lowlands as stated by Fu *et al.* (2005).

and analyses of community composition and vegetation succession) (Song *et al.*, 2008; Wei *et al.*, 2010) have been conducted. However, few studies have addressed genetic differentiation among animal populations on these islands. In addition, many phylogeographic studies have focused on old oceanic islands (> 10 million years) (e.g., Cox *et al.*, 2010) rather than those with a short isolation time (Wei *et al.*, 2008). Thus, there are few reports about genetic analyses of the animals on Zhoushan Archipelago. Cai *et al.* (2012) reported that some populations of *Takydromus septentrionalis* from Zhoushan Archipelago formed independent clades. However, Lin *et al.* (2008) indicated there was no significant genetic divergence among the Chinese cobra *(Naja atra)* population on the largest island of Zhoushan Archipelago and another two *Naja atra* populations on the nearby continent, which might be due to recent dispersal events.

Bufo gargarizans is one of the most conspicuous species of toads in eastern Asia, and is distributed over a broad region (the lowlands of the Amur River basin, Sakhalin Island through Korea and most regions of China) (Frost, 2016). Recently, several phylogenetic studies (Garcia-Porta *et al.*, 2012; Igawa *et al.*, 2006; Recuero *et al.*, 2012) and intraspecific phylogenetic studies of *Bufo* species have been conducted (Fu *et al.*, 2005; Hu *et al.*, 2007; Liu *et al.*, 2000; Macey *et al.*, 1998; Yu *et al.*, 2014). However, they have not included the *Bufo* species from Zhoushan Archipelago. Thus, the affinities and diversities of these species on Zhoushan Archipelago remains unknown.

In this work, we aim to examine the genetic origin and genetic divergence of *B. gargarizans* populations on Zhoushan Archipelago. We used a large amount of mtDNA sequences of *B. gargarizans* collected throughout Zhoushan Archipelago and its adjacent continent as well as some published DNA sequences of *B. gargarizans* from other major continental regions in China. Then, phylogenetic research on this species was conducted. In addition, detailed demographic and dating analyses of *B. gargarizans* were initially performed to analyze population divergence and exchange between *B. gargarizans* from Zhoushan islands and nearby mainland areas.

2. Materials and Methods

2.1 Sampling and tissue collection In this paper, 226 specimens were used (Appendix I). 155 specimens were newly collected from Zhoushan Archipelago and its nearby coastal areas. The rest (77) were continental specimens in Fu *et al.* (2005), and the sequences of those were retrieved from GenBank (ND1-ND2: AY937840-76; Control Region (CR): AF190230-31, AF190233-35, AF190254-55, AY924310-73). Specimens or tissue samples were stored in 100% alcohol immediately after collection.

2.2 Laboratory procedures DNA was extracted using a commercial Kit. The ND1-ND2 fragment was amplified using the primers L4437: 5'- CGA GCA TCC TAC CCA CGA TTT CG -3' and H4980: 5'- ACT TTT CGG ATT TGA GTT TGR TT -3' (Macey *et al.*, 1998). The primers used to amplify the CR fragment were Cytb2L: 5'- TGG GGA CAA ATA TCC TTC TGG GG -3' and ControlB: 5'- GTC CAT TGG AGG TTA AGA TCT ACC A -3' (Goebel *et al.*, 1999). In addition, standard PCR condition and protocol were adopted, and all PCR products were commercially sequenced.

2.3 Phylogenetic analyses and divergence time estimation Sequences were initially aligned using Clustal X. Then they were checked and revised manually in MEGA 6.0. Bayesian MCMC method implemented within BEAST v1.8.2 (Drummond *et al.*, 2012) was used to infer gene trees. The DNA substitution models that incorporated rate variations were used to calculate within-locus heterogeneity. The mtDNA was partitioned into four parts: codon positions 1 and 2 (cp1 and cp2), codon position 3 (cp3), tRNA, and Control Region (CR). Models of DNA substitution were determined using MODELTEST v 3.7. *Torrentophryne aspinia* and *Torrentophryne tuberospinia* (i.e. *Bufo aspinius* and *Bufo tuberospinius*, respectively, Frost, 2016) sequences were used as outgroups (Liu *et al.*, 2000). A partition homogeneity test was performed to quantify the phylogenetic incongruence among different genes (1000 replications) (Farris *et al.*, 1995) in PAUP* (Swofford, 1998). Macey *et al.* (1998) estimated an evolutionary rate of 0.69% pairwise difference per lineage per million years for the ND1-ND2 fragment of the *Bufo bufo* complex (including *B. gargarizans* species group), using the geographical event that separated the European and Asian stock of the complex. According to the rates published by Macey *et al.* (1998), we applied a single strict clock and used a LogNormal (0.0069, 0.185) prior on the clock rate with a standard deviation of 0.185 substitutions per site per million years to reflect uncertainty in the rate.

2.4 The population genetic cluster and historical demographic analyses A Bayesian approach was adopted with a stochastic optimization algorithm for analyzing population structure (Corander *et al.*, 2003, 2008). Bayesian Analysis of Population Structure (BAPS) software v6.0 (Corander *et al.*, 2008; Corander and Tang, 2007) was used, and genetically similar individuals were clustered into panmictic groups. BAPS software was run with both fragments setting different number of clusters (up to 20), depending on the scenario of sampling.

To evaluate the historical movements and population growth of *B. gargarizans*, a mismatch distribution, Tajima's *D* (Tajima, 1989) and Fu's *F* tests of selective neutrality (Fu, 1997) were conducted in Arlequin v3.5 using default settings, with 1000 simulations. A Bayesian skyline plot (BSP) was used to assess changes in the effective breeding population size over time, using BEAST 1.8.2 (Drummond *et al.*, 2012). The ND1–ND2 sequence data were analyzed under a coalescent Bayesian skyline tree model and a relaxed (uncorrelated lognormal) molecular clock (Drummond *et al.*, 2006), using the fixed rate of 0.69% substitutions per lineage per million years. However, potential population structure may cause the decline in effective population size estimated by the BSP analysis (Heller *et al.*, 2013). Thus, BSP analyses were conducted on individual clades to reduce the confounding effect of population structure caused by potential violations of panmixia assumption in BSP inferences.

2.5 Ethical Approval and Informed Consent Fieldwork and tissue sampling authorization was provided by the Zhejiang Province Forestry Bureau. All experimental protocols were performed in accordance with guidelines from the China Council on Animal Care and the Ethics Committee of Animal Experiments at China Jiliang University.

3. Results

3.1 Sequence characteristics Sequencing of PCR products of *B. gargarizans* provided 1436 bp mtDNA, including a ND1–ND2 fragment of 815 bp in length and a Cytb–Control Region (CR) portion of 621 bp in length. After partition, we obtained the cp1 and cp2 404/30, cp3 202/100, tRNAs 209/2 and CR 621/215 (sequence alignment lengths/numbers of variable positions).

The TrN + I model was used for cp1 and cp2, the GTR + G model was used for cp3, the TrN model was used for tRNAs, and the TrN + I + G model was used for CR. 176 sequences of ND1–ND2 and 179 sequences of Cytb–CR representing 226 individuals were included into the analysis. Our sequences have been deposited in GenBank (Accession Nos.: KY806413–KY806551, KY806302–KY806409, Appendix I)

3.2 The population structures and phylogenetics The homogeneity test showed there was no significant heterogeneity among genes ($P = 0.737$), indicating it was reasonable to combine them into one analysis. BEAST analyses using ND1–ND2 and Cytb–CR fragments showed seven clades of *B. gargarizans* (Figures 1 and 2). The basal clade A (red) in the chronogram of the species included individuals generally from western China except two individuals from the nearby central region (Figure 2). It is consistent with the point that the West Highland is the origin of this species.

There was notable subsequent divergence in three clades. The first split represented divergence of populations from the northeast region (clade B including two individuals, orange). The second split represented divergence of populations from the central and southeast continental region (clade C, yellow). The third split represents divergence of populations (clade D, green) from northeast, central and west regions of China. Among the three most recent clades (E, F and G), clade E was represented by a specimen from Taiwan. Clades F included specimens from the Zhoushan Archipelago, and Clades G included specimens from the Zhoushan Archipelago and the southeastern continental region (two) (Figure 2 and Appendix II).

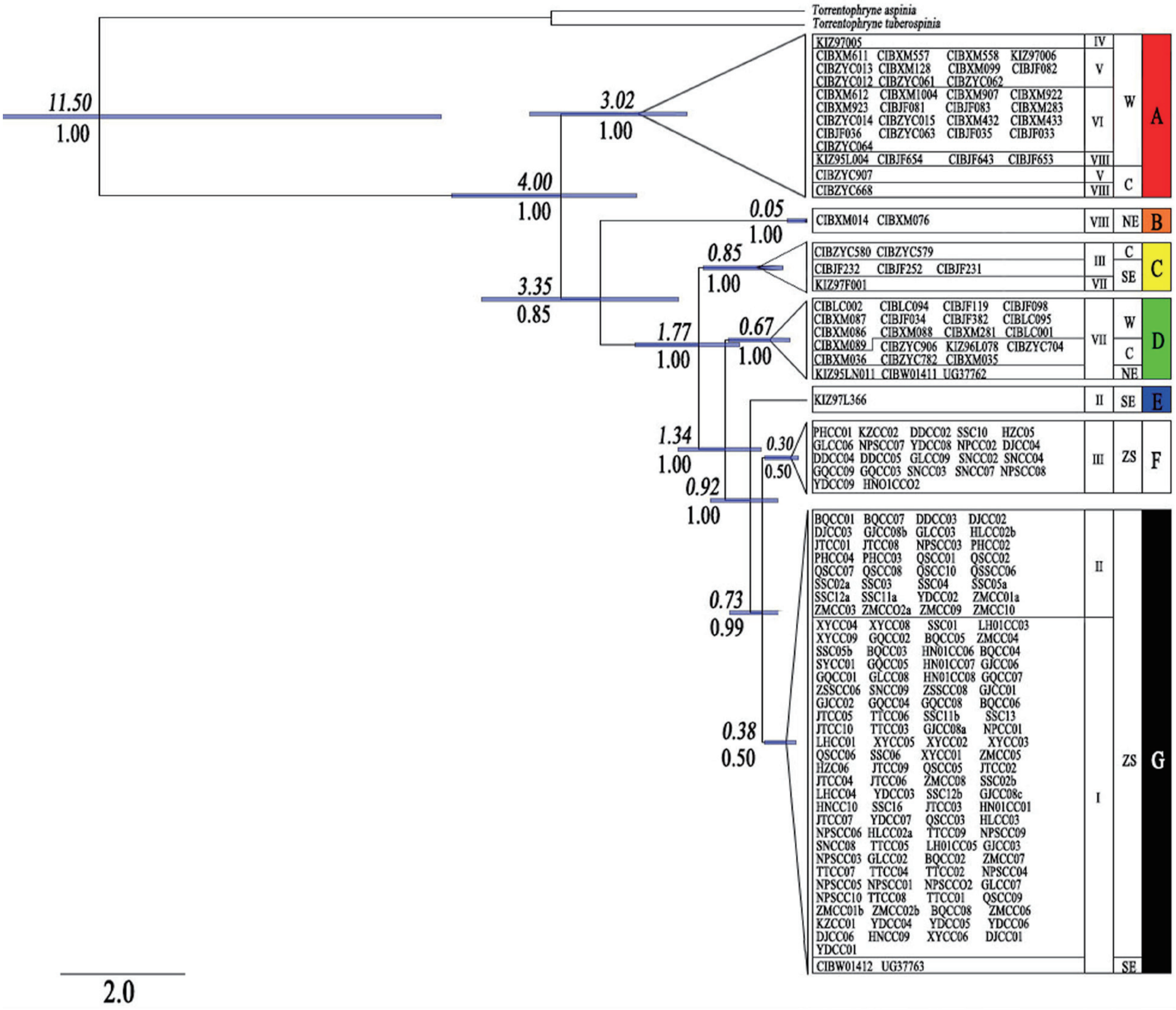
Population structures estimated by BAPS were generally similar with clades: structure I and II in clade G (except that clade E included one specimen of II), structure III in clade F (except that clade C included five specimens of III), and structure VII in clade D (except that clade C included one specimen of VII). However, for specimens in clades A, B and C, there was significant difference in population structure (Figure 2).

3.3 Divergence time estimation The posterior node age corresponding to the divergence into highland and lowland clades averaged 4.00 million years (95% HPD 3.35-4.69 mya) (Figure 2). The earliest divergence within the highland clade (95% HPD 2.41-3.62 mya, 3.02 mya) appeared to just postdate the divergence in the lowland clade (95% HPD 2.66-4.06 mya, 3.35 mya) (Figure 2). Within the lowland clade, two divergence events occurred at 1.77 and 1.34 mya, respectively, and three more recent splits occurred in the late Pleistocene (Figure 2). All early divergence events within the highland major clades appeared to be associated with the middle Pliocene.

3.4 Historical demographic analyses Non-significant negative values of Fu's F and Tajima's D indicated that no clades had undergone significant recent demographic expansion, though clade G had a great significant negative Fu's F value and a marginally significant negative Tajima's D value ($P = 0.054$). BSP analyses of five major clades (i.e. clades including at least five specimens) suggested that the population growth of the clades D, F and G began to slightly accelerate approximately 0.20, 0.02 and 0.02 mya, respectively (Figure 3). The growth of clade G was the most obvious and consistent with the significant Tajima's D demographic analyses (Table 1).

4. Discussion

Although topological patterns obtained in this paper were similar to those in previous studies (Macey *et al.*, 1998; Fu *et al.*, 2005; Hu *et al.*, 2007), there were some differences we should pay attention to. In this paper, we found two major distinct clades (clade A and clades B-G) primarily corresponding to specimens from west highlands (except two specimens distributed in nearby central region) and east lowlands, respectively (Figure 1). The highland clades were different from the basal paraphyletic groups of west highland populations in Fu *et al.* (2005), though both topologies indicated the west origin of species due to the basal clades were geographically represented by west highlands. Macey *et al.* (1998) suggested an early intraspecific division of *B. gargarizans* (Figure 1) following dispersion of populations from east lowlands to west highlands, and Fu *et al.* (2005) suggested dispersal, instead of vicariance, dominated the history of the species. In contrast, we concluded that the vicariance-dispersal hypothesis associated with the earliest west highland-east lowland divergence within the species cannot be rejected. Clade D (green) was distributed not only in central and northeast region of China but also in seven sites in western regions


11.50
1.00
3.02
1.00
4.00
1.00
0.05
1.00
0.85
1.00
3.35
0.85
1.77
1.00
0.67
1.00
1.34
1.00
0.30
0.50
0.92
1.00
0.73
0.99
0.38
0.50
2.0
Torrentophryne aspinia
Torrentophryne tuberospinia
KIZ97005 IV
CIBXM611 CIBXM557 CIBXM558 KIZ97006
CIBZYC013 CIBXM128 CIBXM099 CIBJF082
CIBZYC012 CIBZYC061 CIBZYC062 V
CIBXM612 CIBXM1004 CIBXM907 CIBXM922
CIBXM923 CIBJF081 CIBJF083 CIBXM283
CIBZYC014 CIBZYC015 CIBXM432 CIBXM433
CIBJF036 CIBZYC063 CIBJF035 CIBJF033
CIBZYC064 VI
KIZ95L004 CIBJF654 CIBJF643 CIBJF653 VIII
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A
CIBXM014 CIBXM076 VIII NE B
CIBZYC580 CIBZYC579
CIBJF232 CIBJF252 CIBJF231 III
KIZ97F001 VII
C
SE
C
CIBLC002 CIBLC094 CIBJF119 CIBJF098
CIBXM087 CIBJF034 CIBJF382 CIBLC095
CIBXM086 CIBXM088 CIBXM281 CIBLC001
CIBXM089 CIBZYC906 KIZ96L078 CIBZYC704
CIBXM036 CIBZYC782 CIBXM035
KIZ95LN011 CIBW01411 UG37762
VII
W
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D
KIZ97L366 II SE E
PHCC01 KZCC02 DDCC02 SSC10 HZC05
GLCC06 NPSCC07 YDCC08 NPCC02 DJCC04
DDCC04 DDCC05 GLCC09 SNCC02 SNCC04
GQCC09 GQCC03 SNCC03 SNCC07 NPSCC08
YDCC09 HNO1CCO2
III ZS F
BQCC01 BQCC07 DDCC03 DJCC02
DJCC03 GJCC08b GLCC03 HLCC02b
JTCC01 JTCC08 NPSCC03 PHCC02
PHCC04 PHCC03 QSCC01 QSCC02
QSCC07 QSCC08 QSCC10 QSSCC06
SSC02a SSC03 SSC04 SSC05a
SSC12a SSC11a YDCC02 ZMCC01a
ZMCC03 ZMCCO2a ZMCC09 ZMCC10
II
XYCC04 XYCC08 SSC01 LH01CC03
XYCC09 GQCC02 BQCC05 ZMCC04
SSC05b BQCC03 HN01CC06 BQCC04
SYCC01 GQCC05 HN01CC07 GJCC06
GQCC01 GLCC08 HN01CC08 GQCC07
ZSSCC06 SNCC09 ZSSCC08 GJCC01
GJCC02 GQCC04 GQCC08 BQCC06
JTCC05 TTCC06 SSC11b SSC13
JTCC10 TTCC03 GJCC08a NPCC01
LHCC01 XYCC05 XYCC02 XYCC03
QSCC06 SSC06 XYCC01 ZMCC05
HZC06 JTCC09 QSCC05 JTCC02
JTCC04 JTCC06 ZMCC08 SSC02b
LHCC04 YDCC03 SSC12b GJCC08c
HNCC10 SSC16 JTCC03 HN01CC01
JTCC07 YDCC07 QSCC03 HLCC03
NPSCC06 HLCC02a TTCC09 NPSCC09
SNCC08 TTCC05 LH01CC05 GJCC03
NPSCC03 GLCC02 BQCC02 ZMCC07
TTCC07 TTCC04 TTCC02 NPSCC04
NPSCC05 NPSCC01 NPSCCO2 GLCC07
NPSCC10 TTCC08 TTCC01 QSCC09
ZMCC01b ZMCC02b BQCC08 ZMCC06
KZCC01 YDCC04 YDCC05 YDCC06
DJCC06 HNCC09 XYCC06 DJCC01
YDCC01
CIBW01412 UG37763
I
ZS
SE
G

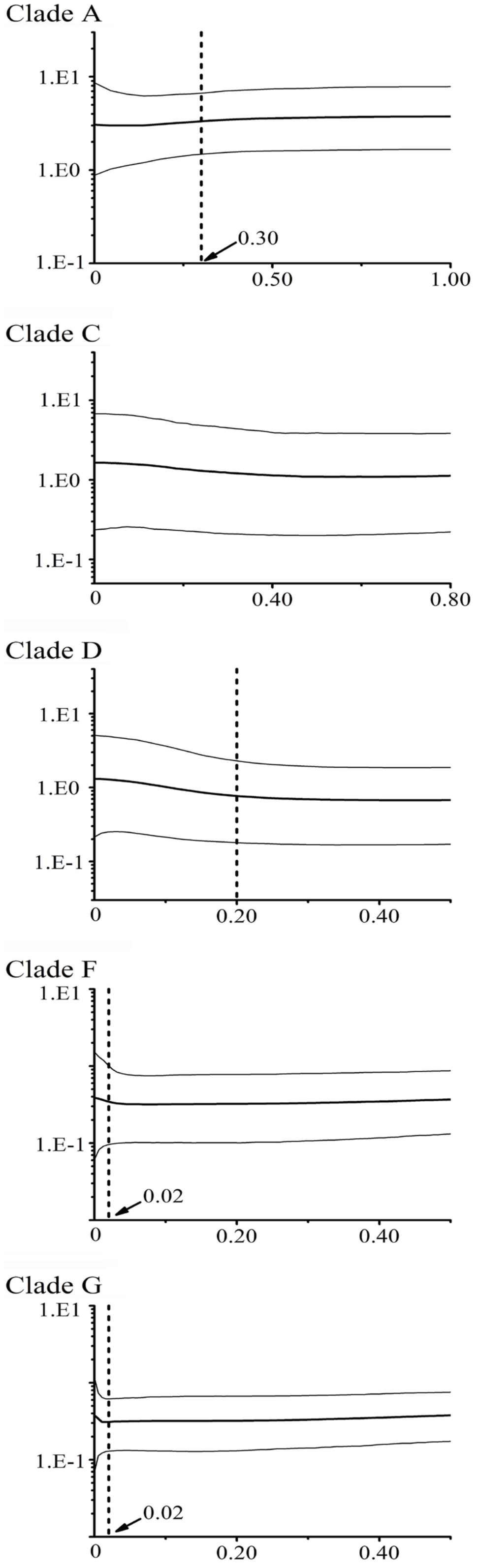
Figure 2 Species tree chronogram for *B. gargarizans* from a combined four partitioned mtDNA dataset generated on BEAST. Clades (A-G) corresponded to the solid colored circles in Figure 1. Detailed information of geographical areas and labeling of the samples were shown in appendix I (W, C, NE, SE and ZS denoted west, central, northeast, southeast and Zhoushan Archipelago, respectively). The values at nodes showed the mean divergence time (above) and the posterior probability (below). Eight population structures (I-VIII) represented different groups of individuals.

of China (including sites 17–20 in the west highlands, and sites 8, 10, and 13 near these highlands, Figure 1). This indicated that dispersal occurred much later than vicariance, and supported the hypothesis of dispersal from low elevation to high elevation (Macey *et al.*, 1998). The result did not contradict that of Fu *et al.* (2005), which also indicated a trend of dispersal from west to east for some lineages. The result also did not contradict the conclusion that dispersal instead of vicariance might have occurred multiple times (Hu *et al.*, 2007). In fact, the mtDNA tree in Figure 2 implied that other multiple dispersal events in low-elevation regions were also possible.

After the split that generated clade D, three subsequent clades appeared within this lineage. This supported the hypothesis that the most recent splits occurred in the southeast region. In addition, among these clades, clade E occurred first, then clades F and G appeared as sister clades on the Zhoushan Archipelago. This supported the hypothesis that divergence events occurred within populations on Zhoushan Archipelago and populations from the continental region. Dispersion after vicariance could also occurred since two specimens in clade G were shown to be from the southeast region. This was also supported by demographic analyses of Tajima's *D*, which showed significant and rapid range expansion of clade G (Table 1).

The first split event occurred at 4.0 mya (95% HPD

3.35-4.69 mya) (Figure 2), later than that in Macey *et al.* (1998). The posterior interval was also narrower than that estimated by them, which was 5.0-10 mya. This period of 3.35-4.69 mya overlapped the extensive uplift period of Tibetan plateau in the early Pliocene (Shi *et al.*, 1998). Although the uncertainty existed over the time of geological events in the Qinghai-Tibetan Plateau (Renner, 2016), there was clear evidence of spatial relationships between major clade distributions and some major features of different elevational regions. The earliest divergence within the highland clade of *B. gargarizans* occurred at 3.02 mya (95% HPD 2.41-3.62 mya) (Figure 2). The formation of many mountain ranges that surround the southeastern Qinghai-Tibetan Plateau (QTP), e.g. uplift of the Henduan mountains, intensified during the Qingzang Movement beginning at 3.40 mya (Qian and Chen, 1984; Shi *et al.*, 1998). These clades became isolated within the mountainous basins at this time. Phylogenetic studies of endemic animals on the QTP, including several birds (Bao *et al.*, 2010; James *et al.*, 2003; Qu *et al.*, 2006), frogs (Li *et al.*, 2012) and mammals (Fan *et al.*, 2012; Luo *et al.*, 2004), all revealed that many splits within closely related species could be traced back to this period.


Clade A

Clade C

Clade D

Clade F

Clade G

Figure 3 Bayesian skyline plot showing the growth history of *B. gargarizans* population. Horizontal axis: time in 10^6 years before present; vertical axis: estimated population size [units = $N_e \tau$, the product of effective population size and generation length in years (log transformed)]. The dotted lines indicated the time of expansion or shrinkage of populations.

The earliest divergences within the lowland clade occurred at 3.35 mya (95% HPD 2.66-4.06 mya) (Figure 2). Two subsequent divergence events (1.77 and 1.34 mya) were also dated to the early Pleistocene. At the beginning of the late Pleistocene, three more recent split events occurred. These split events seemed to have occurred outside of the QTP, but were still associated with the first (3.40 mya) and third (1.70 mya) (Figure 2) stages of the Qiangzang Movement within the QTP. Climatic changes influenced by these uplift events (Pan *et al.*, 1998; Zheng and Yao, 2006) might have contributed to the divergence of these clades. Clade E occurred at 0.92 mya (95% HPD 0.67-1.17 mya) (Figure 2) and the clade F/G occurred on Zhoushan Archipelago when the sea level gradually rose during the interglaciation period before the penultimate glaciation (0.72-0.59 mya) (Li, 2008).

Population sizes of the Zhoushan archipelago clades F and G began to increase until 0.02 mya (Figure 3), corresponding to the late stage of Last Glaciation (0.023-0.010 mya) when the sea levels fell (Li, 2008). Population size of Clade D in the northeast, central and western regions increased until the beginning of the interglacial period (approximately 0.13 mya), though the increase in population size of clades D and F was not significant in demographic analyses (Table 1). In addition,

Table 1 Haplotype diversity, nucleotide diversity, Fu's *F*, Tajima's *D* and raggedness indices estimated from ND1–ND2 sequences in six clades of *B. gargarizans*.

Clades	n	Unique haplotypes	Haplotype diversity	Nucleotide diversity	Fu's *F*	Tajima's *D*	Raggedness index (*P* value)	Tau
A	18	14	0.96 ± 0.03	0.0332 ± 0.0173	–0.79 (0.34)	–0.56 (0.30)	0.03 (0.30)	30.67
B	2	2	1.00 ± 0.50	0.0019 ± 0.0026	0.00 (0.24)	0.00 (1.00)	0.00 (0.00)	0
C	5	5	1.00 ± 0.12	0.0098 ± 0.0065	–0.96 (0.16)	0.80 (0.76)	0.20 (0.64)	10.28
D	10	7	0.86 ± 0.10	0.0069 ± 0.0041	–0.68 (0.32)	–0.44 (0.34)	0.08 (0.47)	11.04
F	22	6	0.69 ± 0.08	0.0029 ± 0.0018	0.42 (0.62)	–0.94 (0.17)	0.25 (0.08)	3.66
G	119	12	0.69 ± 0.03	0.0019 ± 0.0013	–2.98 (0.12)	–1.38 (0.05)	0.05 (0.09)	1

the population size of highland clade A have slightly decreased since 0.30 mya (Figure 3). Possibly, Pleistocene glaciations caused not only the expansion/shrinkage of size of continent populations during interglacial/glacial periods, but also the expansion of size of island population due to the falling of sea level.

To date, despite many ecological studies about the population of frog on island (Li *et al.*, 2006; Wu *et al.*, 2006; Xu *et al.*, 2013), population genetic diversity and phylogeography of amphibian species from the Zhoushan Archipelago have received little attention. Some research indicated that genetic diversity of *Pelophylax nigromaculatus* on the island was lower than that on mainland (Wang *et al.*, 2014). It is consistent with our result. The haplotype diversity and nucleotide diversity of the continental clades A, B, C, and D were higher than those of the island subclades F and G.

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Appendix I Detailed specimens and sampling information.

Labeling of the samples	Localities	Latitude	Longitude	Geographical area	Genebank Accession ND1–ND2	Genebank Accession Cytb–CR	Samples used
KIZ91A089	Tengchong Co.; Yunnan,Chian	-	-	W	-	AF190255	Liu *et al.* (2000)
CIBXM611	(3)Derong	28.7	99.2	W	AY936865	AY924311	Fu *et al.* (2005)
CIBXM612	(3)Derong	28.7	99.2	W	-	AY924313	Fu *et al.* (2005)
KIZ93A011	Yangbi Co.; Yunnan,Chian	-	-	W	-	AF190254	Liu *et al.* (2000)
CIBLC094	(20)Baiyu	30.99	99.72	W	-	AY924372	Fu *et al.* (2005)
CIBLC095	(20)Baiyu	30.99	99.72	W	-	AY924373	Fu *et al.* (2005)
KIZ97005	(2)Zhongdian	27.7	99.7	W	AY936840	AY924310	Fu *et al.* (2005)
CIBJF382	(18)Luhou	31.4	100.62	W	AY936862	AY924369	Fu *et al.* (2005)
CIBLC001	(19)Daofu	30.49	101.47	W	-	AY924371	Fu *et al.* (2005)
CIBLC002	(19)Daofu	30.49	101.47	W	-	AY924370	Fu *et al.* (2005)
CIBJF098	(17)Xinduqiao	30.03	101.47	W	AY936861	AY924368	Fu *et al.* (2005)
CIBXM1004	(7)Jiulong	29	101.5	W	-	AY924339	Fu *et al.* (2005)
CIBXM557	(6)Mianning	28.5	102.1	W	AY936842	AY924315	Fu *et al.* (2005)
CIBXM558	(6)Mianning	28.5	102.1	W	AY936843	AY924316	Fu *et al.* (2005)
CIBXM907	(5)Yele	28.93	102.19	W	-	AY924314	Fu *et al.* (2005)
KIZ95L004	(1)Kunming	25	102.7	W	AY936866	AF190230	Fu *et al.* (2005)
KIZ97006	(1)Kunming	25	102.7	W	AY936841	AY924312	Fu *et al.* (2005)
CIBXM922	(4)Xichang	25.87	102.76	W	-	AY924317	Fu *et al.* (2005)
CIBXM923	(4)Xichang	25.87	102.76	W	-	AY924318	Fu *et al.* (2005)
CIBJF082	(16)Baoxing	30.3	102.8	W	AY936844	AY924319	Fu *et al.* (2005)
CIBJF081	(16)Baoxing	30.3	102.8	W	-	AY924322	Fu *et al.* (2005)
CIBJF083	(16)Baoxing	30.3	102.8	W	-	AY924323	Fu *et al.* (2005)
CIBXM281	(10)Hongya	29.65	102.95	W	AY936857	AY924354	Fu *et al.* (2005)
CIBXM283	(10)Hongya	29.65	102.95	W	-	AY924328	Fu *et al.* (2005)
CIBZYC012	(14)Qionglai	30.25	103.09	W	AY936846	AY924330	Fu *et al.* (2005)
CIBZYC013	(14)Qionglai	30.25	103.09	W	AY936845	AY924329	Fu *et al.* (2005)
CIBZYC014	(14)Qionglai	30.25	103.09	W	-	AY924331	Fu *et al.* (2005)
CIBZYC015	(14)Qionglai	30.25	103.09	W	-	AY924332	Fu *et al.* (2005)
CIBXM432	(15)Dayi	30.63	103.17	W	-	AY924326	Fu *et al.* (2005)
CIBXM433	(15)Dayi	30.63	103.17	W	-	AY924327	Fu *et al.* (2005)
UG37431	(8)Omei Mt.-I	29.58	103.28	W	AY936858	AY924356	Fu *et al.* (2005)
CIBXM128	(9)Omei Mt.-II	29.58	103.28	W	AY936869	AY924321	Fu *et al.* (2005)
CIBJF033	(9)Omei Mt.-II	29.58	103.28	W	-	AY924320	Fu *et al.* (2005)
CIBJF035	(9)Omei Mt.-II	29.58	103.28	W	-	AY924324	Fu *et al.* (2005)
CIBJF036	(9)Omei Mt.-II	29.58	103.28	W	-	AY924325	Fu *et al.* (2005)
CIBJF119	(9)Omei Mt.-II	29.58	103.28	W	-	AY924355	Fu *et al.* (2005)
CIBJF034	(9)Omei Mt.-II	29.58	103.28	W	-	AY924357	Fu *et al.* (2005)
CIBXM099	(12)Pengxian	31.23	103.75	W	AY936868	AY924361	Fu *et al.* (2005)
CIBZYC061	(11)Maoxian	31.7	103.88	W	AY936847	AY924333	Fu *et al.* (2005)
CIBZYC062	(11)Maoxian	31.7	103.88	W	AY936848	AY924334	Fu *et al.* (2005)
CIBZYC064	(11)Maoxian	31.7	103.88	W	-	AY924336	Fu *et al.* (2005)
CIBZYC063	(11)Maoxian	31.7	103.88	W	-	AY924335	Fu *et al.* (2005)
CIBXM086	(13)Zhongjiang	31	104.6	W	AY936855	AY924352	Fu *et al.* (2005)
CIBXM087	(13)Zhongjiang	31	104.6	W	AY936856	AY924353	Fu *et al.* (2005)
CIBXM089	(13)Zhongjiang	31	104.6	W	-	AY924337	Fu *et al.* (2005)
CIBXM088	(13)Zhongjiang	31	104.6	W	-	AY924360	Fu *et al.* (2005)
CIBJF643	(21)Wengxian	33.06	104.69	W	AY936849	AY924340	Fu *et al.* (2005)
CIBJF653	(21)Wengxian	33.06	104.69	W	AY936850	AY924341	Fu *et al.* (2005)
CIBJF654	(21)Wengxian	33.06	104.69	W	AY936851	AY924342	Fu *et al.* (2005)
CIBZYC782	(25)Nanchuan	29.07	107.19	C	AY936854	AY924351	Fu *et al.* (2005)
CIBZYC704	(22)Suiyang	28.23	107.3	C	AY936875	AY924359	Fu *et al.* (2005)
CIBZYC579	(24)Zhangjiajie	29.3	110.4	C	AY936871	AY924346	Fu *et al.* (2005)
CIBZYC580	(24)Zhangjiajie	29.3	110	C	AY936872	AY924347	Fu *et al.* (2005)
CIBXM035	(26)Fushun	29.2	105	C	-	AY924358	Fu *et al.* (2005)
CIBXM036	(26)Fushun	29.2	105	C	-	AY924363	Fu *et al.* (2005)
CIBZYC906	(27)Wangyuan	32.06	108.17	C	-	AY924362	Fu *et al.* (2005)
CIBZYC907	(27)Wangyuan	32.06	108.17	C	AY936876	AY924338	Fu *et al.* (2005)

(Continued Appendix I)

Labeling of the samples	Localities	Latitude	Longitude	Geographical area	Genebank Accession ND1–ND2	Genebank Accession Cytb–CR	Samples used
KIZ96L078	(35)Xian	-	-	C	-	AF190235	Fu *et al.* (2005)
CIBZYC668	(23)Jiangkou	27.9	108.72	C	AY936852	AY924343	Fu *et al.* (2005)
CIBW01411	(28)Baihuashan	39.78	115.4	NE	AY936859	AY924365	Fu *et al.* (2005)
CIBW01412	(28)Baihuashan	39.78	115.4	NE	AY936863	AY924364	Fu *et al.* (2005)
UG37763	(29)Jixian	40.1	117.2	NE	AY936864	AY924367	Fu *et al.* (2005)
UG37762	(29)Jixian	40.1	117.2	NE	AY936860	AY924366	Fu *et al.* (2005)
KIZ95LN011	(34)Zhuanghe	-	-	NE	-	AF190233	Fu *et al.* (2005)
CIBXM014	(30)Antu	42.55	128.3	NE	AY936870	AY924344	Fu *et al.* (2005)
CIBXM076	(30)Antu	42.55	128.3	NE	AY936867	AY924345	Fu *et al.* (2005)
KIZ97F001	(32)Guadun	27.73	117.66	SE	-	AF190234	Fu *et al.* (2005)
CIBJF252	(32)Guadun	27.73	117.66	SE	AY936874	AY924350	Fu *et al.* (2005)
CIBJF231	(31)Lin'an	30.2	119.7	SE	AY936853	AY924348	Fu *et al.* (2005)
CIBJF232	(31)Lin'an	30.2	119.7	SE	AY936873	AY924349	Fu *et al.* (2005)
KIZ97L366	(33)Taiwan	-	-	SE	-	AF190231	Fu *et al.* (2005)
BQCC01	BQ	30.07	122.16	ZS	KY806451	-	In this study
BQCC02	BQ	30.07	122.16	ZS	KY806448	KY806330	In this study
BQCC03	BQ	30.07	122.16	ZS	KY806446	KY806328	In this study
BQCC04	BQ	30.07	122.16	ZS	KY806445	KY806327	In this study
BQCC05	BQ	30.07	122.16	ZS	KY806450	KY806331	In this study
BQCC06	BQ	30.07	122.16	ZS	KY806447	KY806329	In this study
BQCC07	BQ	30.07	122.16	ZS	KY806449	-	In this study
BQCC08	BQ	30.07	122.16	ZS	-	KY806332	In this study
DDCC02	DD	30.3	122.19	ZS	KY806454	KY806335	In this study
DDCC03	DD	30.3	122.19	ZS	KY806455	-	In this study
DDCC04	DD	30.3	122.19	ZS	KY806452	KY806333	In this study
DDCC05	DD	30.3	122.19	ZS	KY806453	KY806334	In this study
DJCC01	DJ	30.03	122.03	ZS	KY806420	KY806307	In this study
DJCC02	DJ	30.03	122.03	ZS	KY806422	-	In this study
DJCC03	DJ	30.03	122.03	ZS	KY806423	-	In this study
DJCC04	DJ	30.03	122.03	ZS	KY806424	-	In this study
DJCC06	DJ	30.03	122.03	ZS	KY806421	KY806308	In this study
GJCC01	GJ	29.87	121.98	ZS	KY806416	KY806304	In this study
GJCC02	GJ	29.87	121.98	ZS	KY806417	KY806305	In this study
GJCC03	GJ	29.87	121.98	ZS	KY806414	KY806302	In this study
GJCC06	GJ	29.87	121.98	ZS	KY806415	KY806303	In this study
GJCC08a	GL	30.12	103.09	ZS	-	KY806399	In this study
GJCC08b	GJ	29.87	121.98	ZS	KY806418	-	In this study
GJCC08c	GJ	29.88	121.98	ZS	-	KY806306	In this study
GLCC02	GL	30.12	103.09	ZS	KY806533	KY806398	In this study
GLCC03	GL	30.12	103.09	ZS	KY806534	-	In this study
GLCC06	GL	30.12	103.09	ZS	KY806535	-	In this study
GLCC07	GL	30.12	120.52	ZS	KY806532	KY806397	In this study
GLCC08	GL	30.31	120.38	ZS	KY806531	KY806396	In this study
GLCC09	GL	30.12	103.09	ZS	KY806536	-	In this study
GQCC01	GQ	30.72	122.77	ZS	KY806523	KY806389	In this study
GQCC02	GQ	30.72	122.77	ZS	KY806524	KY806390	In this study
GQCC03	GQ	30.72	122.77	ZS	KY806522	KY806388	In this study
GQCC04	GQ	30.72	122.77	ZS	KY806525	KY806391	In this study
GQCC05	GQ	30.72	122.77	ZS	KY806526	KY806392	In this study
GQCC07	GQ	30.72	122.77	ZS	KY806521	KY806387	In this study
GQCC08	GQ	30.72	122.77	ZS	KY806527	KY806393	In this study
GQCC09	GQ	29.87	122.77	ZS	KY806419	-	In this study
HLCC02a	HL	30.73	122.81	ZS	-	KY806359	In this study
HLCC02b	HL	30.73	122.81	ZS	KY806530	-	In this study
HLCC03	HL	30.73	122.81	ZS	KY806487	KY806354	In this study
HN01CC01	HN	30.85	122.69	ZS	-	KY806386	In this study
HN01CC02	HN	30.85	122.69	ZS	KY806520	-	In this study

(Continued Appendix I)

Labeling of the samples	Localities	Latitude	Longitude	Geographical area	Genebank Accession ND1–ND2	Genebank Accession Cytb–CR	Samples used
HN01CC06	HN	30.85	122.69	ZS	KY806518	KY806384	In this study
HN01CC07	HN	30.85	122.69	ZS	KY806517	KY806383	In this study
HN01CC08	HN	30.85	122.69	ZS	KY806516	KY806382	In this study
HNCC09	HN	30.85	122.69	ZS	KY806519	KY806385	In this study
HNCC10	HN	30.85	122.69	ZS	KY806515	KY806381	In this study
HZC05	HZ	30.12	103.09	ZS	KY806537	-	In this study
HZC06	HZ	30.31	120.38	ZS	KY806457	KY806337	In this study
JTCC01	JT	30.01	121.87	ZS	KY806551	-	In this study
JTCC02	JT	30.01	121.87	ZS	KY806545	KY806404	In this study
JTCC03	JT	30.01	121.87	ZS	KY806549	KY806408	In this study
JTCC04	JT	30.01	121.87	ZS	KY806546	KY806405	In this study
JTCC05	JT	30.01	121.87	ZS	KY806543	KY806402	In this study
JTCC06	JT	30.01	121.87	ZS	KY806547	KY806406	In this study
JTCC07	JT	30.01	121.87	ZS	KY806550	KY806409	In this study
JTCC08	JT	30.01	121.87	ZS	KY806413	-	In this study
JTCC09	JT	30.01	121.87	ZS	KY806548	KY806407	In this study
JTCC10	JT	30.01	121.87	ZS	KY806544	KY806403	In this study
KZCC01	KZ	30	122.24	ZS	KY806456	KY806336	In this study
KZCC02	KZ	30	122.24	ZS	KY806458	-	In this study
LH01CC03	LH	30.82	122.63	ZS	KY806513	KY806377	In this study
LH01CC05	LH	30.82	122.63	ZS	KY806514	KY806378	In this study
LHCC01	LH	30.82	122.63	ZS	-	KY806379	In this study
LHCC04	LH	30.82	122.63	ZS	-	KY806380	In this study
NPCC01	NP	30.27	122.14	ZS	KY806433	KY806317	In this study
NPCC02	NP	30.27	122.14	ZS	KY806434	KY806318	In this study
NPSCC01	NPS	30.27	122.15	ZS	KY806438	KY806322	In this study
NPSCC02	NPS	30.27	122.15	ZS	KY806439	KY806323	In this study
NPSCC03a	NPS	30.27	122.15	ZS	KY806442	-	In this study
NPSCC03b	NPS	30.27	122.15	ZS	-	KY806326	In this study
NPSCC04	NPS	30.27	122.15	ZS	KY806441	KY806325	In this study
NPSCC05	NPS	30.27	122.15	ZS	KY806437	KY806321	In this study
NPSCC06	NPS	30.27	122.15	ZS	KY806435	KY806319	In this study
NPSCC07	NPS	30.27	122.15	ZS	KY806443	-	In this study
NPSCC08	NPS	30.27	122.15	ZS	KY806444	-	In this study
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NPSCC10	NPS	30.27	122.15	ZS	KY806440	KY806324	In this study
PHCC01	PH	30.61	121.05	ZS	KY806539	KY806401	In this study
PHCC02	PH	30.61	121.05	ZS	KY806542	-	In this study
PHCC03	PH	30.61	121.05	ZS	KY806541	-	In this study
PHCC04	PH	30.61	121.05	ZS	KY806540	-	In this study
QSCC01	QS	30.44	122.35	ZS	KY806479	-	In this study
QSCC02	QS	30.44	122.35	ZS	KY806480	-	In this study
QSCC03	QS	30.44	122.35	ZS	KY806476	KY806348	In this study
QSCC05	QS	30.44	122.35	ZS	KY806475	KY806347	In this study
QSCC06	QS	30.44	122.35	ZS	-	KY806350	In this study
QSCC07	QS	30.44	122.35	ZS	KY806481	-	In this study
QSCC08	QS	30.44	122.35	ZS	KY806482	-	In this study
QSCC09	QS	30.44	122.35	ZS	KY806477	KY806349	In this study
QSCC10	QS	30.44	122.35	ZS	KY806483	-	In this study
QSSCC06	QSS	30.44	122.35	ZS	KY806478	-	In this study
SNCC02	SN	30.45	122.29	ZS	KY806471	-	In this study
SNCC03	SN	30.45	122.29	ZS	KY806472	-	In this study
SNCC04	SN	30.45	122.29	ZS	KY806473	-	In this study
SNCC07	SN	30.45	122.29	ZS	KY806474	-	In this study
SNCC08	SN	30.45	122.29	ZS	KY806469	KY806345	In this study
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SSC01	SS	30.71	122.5	ZS	KY806504	KY806370	In this study

(Continued Appendix I)

Labeling of the samples	Localities	Latitude	Longitude	Geographical area	Genebank Accession ND1–ND2	Genebank Accession Cytb–CR	Samples used
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SSC02b	SS	30.71	122.5	ZS	-	KY806374	In this study
SSC03	SS	30.71	122.5	ZS	KY806510	-	In this study
SSC04	SS	30.71	122.5	ZS	KY806506	-	In this study
SSC05a	SS	30.71	122.5	ZS	KY806511	-	In this study
SSC05b	SS	30.71	122.5	ZS	-	KY806376	In this study
SSC06	SS	30.71	122.5	ZS	-	KY806373	In this study
SSC10	SS	30.71	122.5	ZS	KY806512	-	In this study
SSC11a	SS	30.71	122.5	ZS	KY806507	-	In this study
SSC11b	SS	30.71	122.5	ZS	-	KY806372	In this study
SSC12a	SS	30.71	122.5	ZS	KY806508	-	In this study
SSC12b	SS	30.71	122.5	ZS	-	KY806375	In this study
SSC13	SS	30.71	122.5	ZS	KY806503	KY806369	In this study
SSC16	SS	30.71	122.5	ZS	KY806505	KY806371	In this study
SYCC01	SY	30.12	103.09	ZS	KY806538	KY806400	In this study
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TTCC03	TT	30.44	122.41	ZS	KY806494	KY806360	In this study
TTCC04	TT	30.44	122.41	ZS	KY806500	KY806366	In this study
TTCC05	TT	30.44	122.41	ZS	KY806496	KY806362	In this study
TTCC06	TT	30.44	122.41	ZS	KY806495	KY806361	In this study
TTCC07	TT	30.44	122.41	ZS	KY806501	KY806367	In this study
TTCC08	TT	30.44	122.41	ZS	KY806502	KY806368	In this study
TTCC09	TT	30.44	122.41	ZS	KY806497	KY806363	In this study
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XYCC04	XY	30.64	122.06	ZS	KY806431	KY806315	In this study
XYCC05	XY	30.64	122.06	ZS	KY806428	KY806312	In this study
XYCC06	XY	30.64	122.06	ZS	KY806432	KY806316	In this study
XYCC08	XY	30.64	122.06	ZS	KY806430	KY806314	In this study
XYCC09	XY	30.64	122.06	ZS	KY806429	KY806313	In this study
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YDCC03	YD	30.14	122.35	ZS	KY806484	KY806351	In this study
YDCC04	YD	30.14	122.35	ZS	KY806489	KY806356	In this study
YDCC05	YD	30.14	122.35	ZS	KY806490	KY806357	In this study
YDCC06	YD	30.14	122.35	ZS	KY806491	KY806358	In this study
YDCC07	YD	30.14	122.35	ZS	KY806485	KY806352	In this study
YDCC08	YD	30.14	122.35	ZS	KY806493	-	In this study
YDCC09	YD	30.14	122.35	ZS	KY806486	KY806353	In this study
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ZMCC01b	ZM	30.05	122.26	ZS	-	KY806343	In this study
ZMCC02a	ZM	30.05	122.26	ZS	KY806467	-	In this study
ZMCC02b	ZM	30.05	122.26	ZS	-	KY806344	In this study
ZMCC03	ZM	30.05	122.26	ZS	KY806466	-	In this study
ZMCC04	ZM	30.05	122.26	ZS	KY806461	KY806340	In this study
ZMCC05	ZM	30.05	122.26	ZS	KY806460	KY806339	In this study
ZMCC06	ZM	30.05	122.26	ZS	KY806463	KY806342	In this study
ZMCC07	ZM	30.05	122.26	ZS	KY806462	KY806341	In this study
ZMCC08	ZM	30.05	122.26	ZS	KY806459	KY806338	In this study
ZMCC09	ZM	30.05	122.26	ZS	KY806465	-	In this study
ZMCC10	ZM	30.05	122.26	ZS	KY806464	-	In this study
ZSSCC06	ZSS	30.72	122.81	ZS	KY806529	KY806395	In this study
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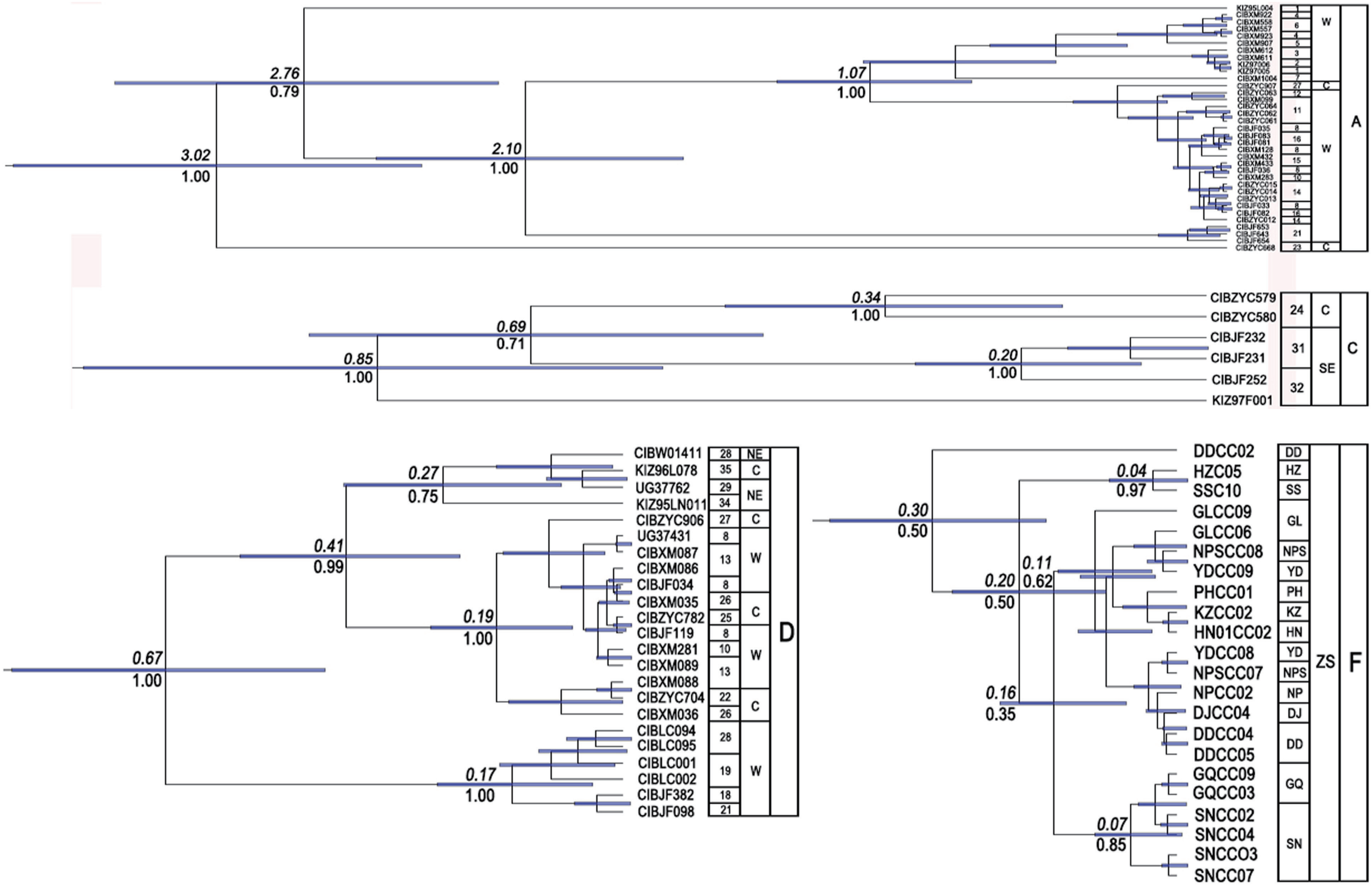
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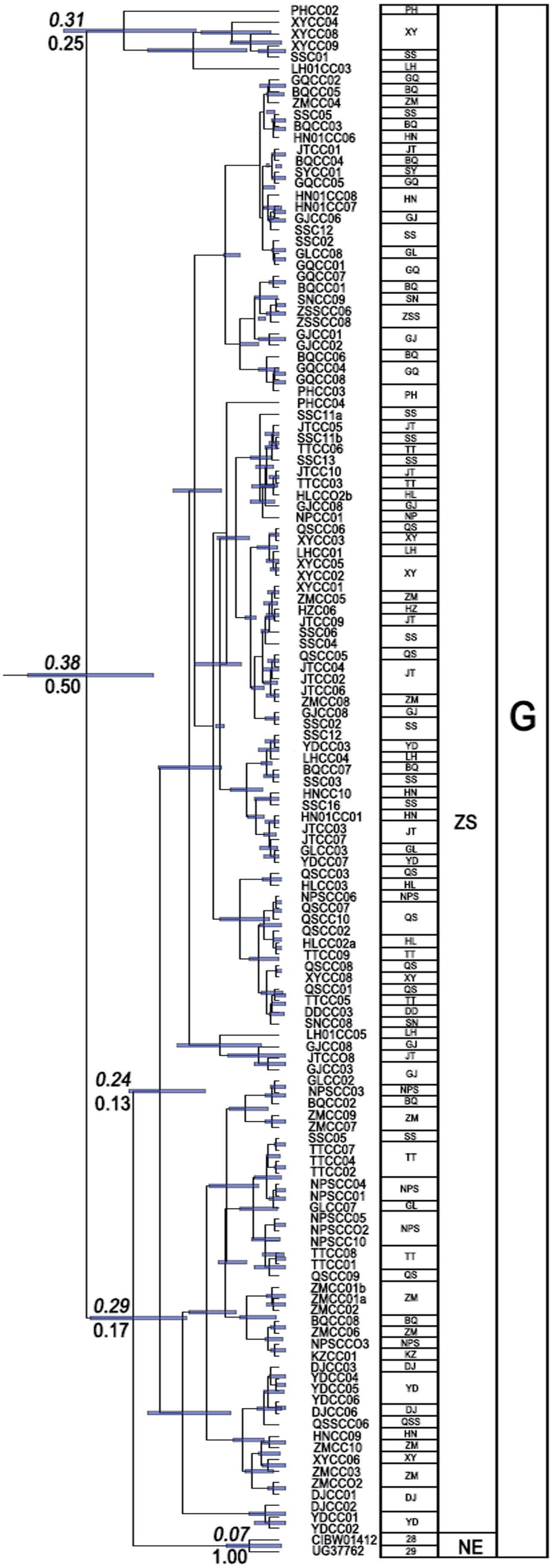

Appendix II Detailed independent clades (A, C, D, F and G) of *B. gargarizans* corresponding to Figure 2. Arabic numbers (1-35) represent the 35 sampling sites based on Fu *et al.* (2005).